THE CHARACTER AND ULTIMATE FATE OF THE LARVAL SALIVARY SECRETION OF PHORMIA REGINA MEIG. (DIPTERA, CALLIPHORIDAE)

HERBERT H. MOOREFIELD AND G. FRAENKEL

Department of Entomology, University of Illinois, Urbana, Illinois

The study of insect salivary glands has now attained a near classic interest, and diverse fundamental researches involving them have been stimulated in many fields. Fraenkel and Brookes (1953) have recently reviewed the literature concerning these glands in Diptera; especially the cytoplasmic changes occurring during the late larval period of *Phormia regina* and several species of *Drosophila,* together with the subsequent passage of cellular components into the lumen of the glands. Various functions have been assigned to this accumulated salivary material and a few postulations have been made regarding its disposition, but it is a noticeable fact that none of the previous workers has offered any experimental evidence as to the fate of the final larval gland contents. Fraenkel and Brookes (1953) have also described the manner in which *Phormia* and certain *Drosophila* orally release a fluid that flows along the underside of the insect, solidifies and securely attaches the newly formed puparium to the surface upon which it rests. By observing the comparative sizes of the salivary glands and investigating the volumes of glandular contents in the larvae before and after this secretion was released, they concluded from this indirect evidence that the secretion produced in the salivary glands was the same material which glued the puparium to its substrate.

The purpose of the present investigation was to collect the secretion from the late larval salivary glands and the material on the exterior of the puparium; to analyze them chromatographically in order to establish the identity of these products and to characterize it as well as the limited quantities would permit.

Methods

Laboratory cultures of adult *Phormia* were maintained on sugar and water. Eggs were collected on pork liver; the larvae were reared in battery jars with moist wood shavings and were furnished fresh liver daily. Prior to pupation, this species leaves its food supply, migrates through the shavings and evacuates its crop. This is referred to as the "empty-crop" stage in this work, and dissections revealed that during this period, the salivary gland lumen contains the greatest quantity of fluid attained throughout the larval life.

The salivary secretion was collected from empty-crop larvae by dissection in a modified Ringer solution (Ephrussi and Beadle, 1936). This was accomplished by cutting off the terminal third of the maggot with scissors, and then manipulating the insect with two pairs of fine forceps to turn it inside out over one of the points of the forceps. With the aid of a dissecting microscope, the glands which previously extended well into the abdomen were then readily discernible, floating free

in the saline, and could be easily teased free of the fat body. They were removed to a fresh solution of Ringer's to be freed of haemolymph, then quickly dipped into a dish of distilled water to remove the salts, and finally transferred to a clean, oversize slide (2 × 3″). Here the glands were punctured with a teasing needle and the secretion permitted to flow out onto the glass. The material from many insects was accumulated on a single slide and pooled by washing it off with distilled water. The volume was reduced in a vacuum desiccator over solid NaOH.

Full grown, empty-crop larvae were placed in petri dishes (about ten insects per dish) to pupate on a layer of clean sand. When the maggots pupated, the material which ordinarily flowed along the underside of their bodies and later anchored them now poured into the sand and merely aggregated a few sand grains at the anterior end of the puparia. By collecting the tanned puparia, carefully chipping off these small clumps of sand grains, pooling them and treating with warm water, the external secretion was extracted.

Hydrolysates were prepared by placing the secretions in small Pyrex tubes, evaporating to dryness in the desiccator, adding 2 ml. of 6 N HCl and sealing off the tubes. These were then steam-autoclaved for 18 hours at 15 lbs. pressure. After hydrolysis, the acid was removed by vacuum desiccation, distilled water added and completely evaporated four times to free the samples of HCl.

The hydrolyzed and raw secretions were analyzed by paper partition chromatography (Consden, Gordon and Martin, 1944), using the ascending modification of Williams and Kirby (1948). Two-dimensional chromatograms were prepared on Whatman No. 1 paper (9 × 11″), using phenol and water (80 gms. and 20 ml.) as the first phase solvent and a water-saturated mixture of equal parts of collidine and lutidine in the second phase. A 0.2% ninhydrin (in water-saturated butanol) spray was used to develop the colors. The final chromatograms were air dried and examined in transmitted light with the aid of a light-box viewer. Resultant spots were identified by, first, Rf values; second, cochromatography (an authentic sample of a known substance is added to the original application on the paper, intensifying the provisionally identified chromatographic spot which then acts as a single entity—behavior as such in different solvents presents strong evidence that the known and unknown are identical materials); and third, specific reactions for some of the components (to be described in a later section).

Results and Discussion

Four separate hydrolysates of the secretion collected from the salivary glands were prepared, and four from the material gathered from larvae which had pupated in the sand. The majority of these preparations were made from insects of different generations. In no cases was there any discrepancy in the number of spots on well prepared chromatograms, and the similar composition of these materials was repeatedly demonstrated by identical patterns consisting of the same components, present in constant relative intensities. The typical pattern, distribution and identification of spots are portrayed in Figure 1.

To test for free amino acids, unhydrolyzed preparations were chromatographed, and a single spot moved off the original site of application. This spot was present in both the glandular secretion and material collected from the puparia; but, when portions of these preparations were first dialyzed and then chromatographed, the

spot was absent. Cochromatography proved the free amino acid to be lysine. Chromatograms of dialyzed and nondialyzed materials, after hydrolysis, yielded lysine spots in each, but the nondialyzed preparations gave a more intense lysine spot. This indicated that, although lysine was present as a free amino acid, there was also lysine conjugated in the protein molecule.

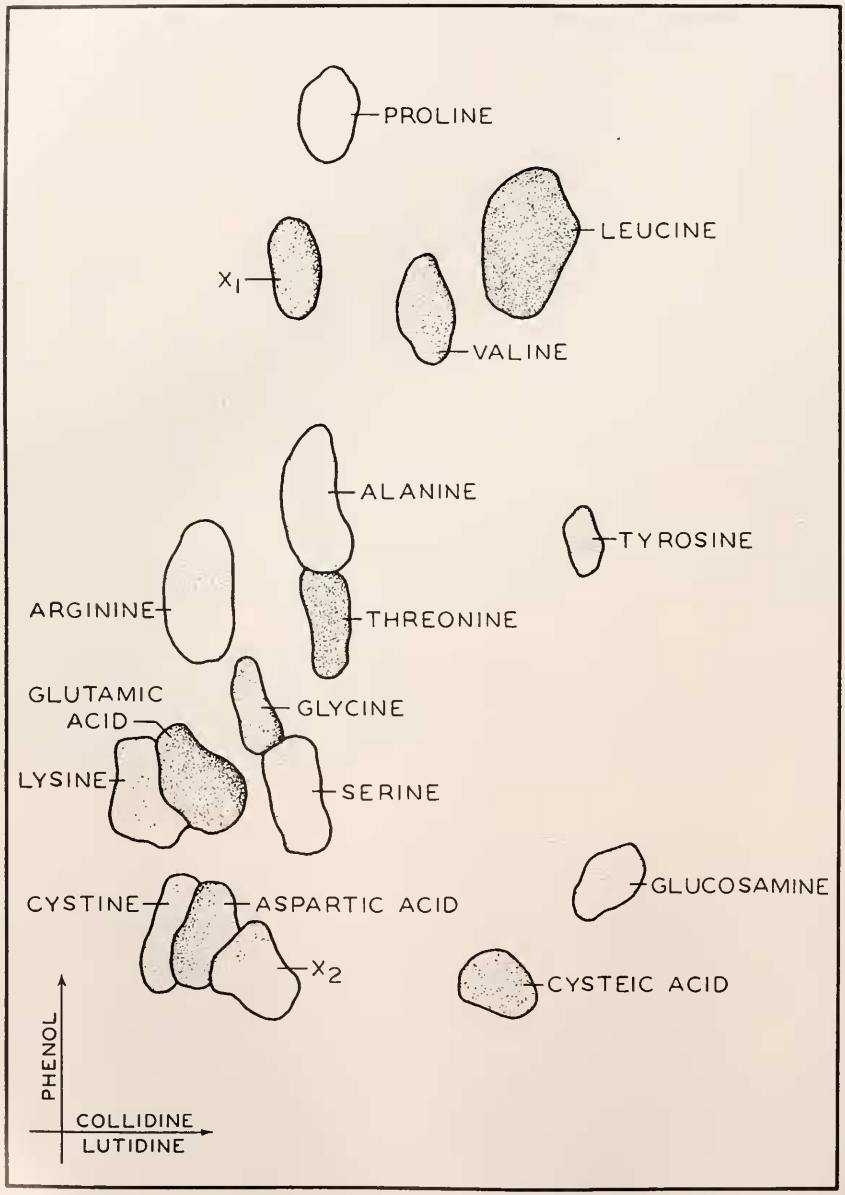

FIGURE 1. Chromatogram of hydrolyzed salivary secretion of larval *Phormia regina*.

Table I presents a complete list of the identified components found in this study together with a compilation of reported analyses made on dipterous salivary glands and associated products.

Cystine and its oxidation product, cysteic acid, appear brown when sprayed with ninhydrin, and both were present in the salivary material. Dent (1947) suggested that cysteic acid may be produced by secondary decomposition during chromatography; however, a sample of cystine hydrolyzed alone and treated as were the salivary products showed but a single cystine spot when chromatographed.

TABLE I

Comparison of chromatographic analyses of dipterous salivary glands and associated products

Component	Salivary gland chromosome *D. melano.* Yasuzumi and Miyao, 1950	Salivary gland chromosome *D. virilis* Blumel and Kirby, 1948	Salivary gland (*in toto*) *D. virilis* Blumel and Kirby, 1948	Salivary gland protein *D. melano.* Kodani, 1948	Salivary gland* secretion *Phormia*
Glycine	+	+	+	+	+
Alanine	+	+	+	+	+
Serine			+	+	+
Threonine			+	+	+
Valine	+	+	+	+	+
Leucine	+	+	+	+	+[1]
Tyrosine			+	+	+
Phenylalanine			+	+	−
Proline	+	+	+	+	+
Aspartic acid	+	+	+	+	+
Glutamic acid	+	+	+	+	+
Arginine	+		+	+	+
Lysine			+	+	+
Cystine					+
Cysteic acid				+	+
Methionine sulfoxide				+[2]	
Glucosamine				+	+
Unidentified			four		two
Free amino acids	none		none[3]		lysine

* Analysis of material from salivary gland and exterior of puparium.

[1] Leucine and/or isoleucine.

[2] Probably in error (Dent, 1948).

[3] LaCour and Drew (1947).

Cystine oxidized with 30% H_2O_2 was used for identification of the cysteic acid spots. Dent (1948) pointed out that by superimposing the peroxide directly on the hydrolyzed material on the paper, cystine would be quantitatively converted to cysteic acid. In chromatograms so treated, the cystine spot did not appear, and the cysteic acid spot was intensified. Further confirmation of cystine was accomplished by spraying the chromatograms with an iodine-azide spray reagent. As demonstrated by Chargaff, Levine and Greene (1948), the sulfur-containing amino acids were revealed as white spots against a brown background. After the location of the spot was marked, and the iodine faded, the paper could be resprayed with ninhydrin.

The spot labeled X_1 occupies the position ascribed by Dent (1947) to methionine sulfoxide and was later reported as such in the salivary secretion of *Drosophila* by Kodani (1948). When methionine was treated with 30% H_2O_2, mixed oxidation products of methionine sulfoxide and methionine sulfone resulted, and when this mixture was cochromatographed with the salivary secretion, X_1 was reinforced by methionine sulfoxide. However, neither adding the peroxide to the original material on the paper before chromatography, nor treating the final chromatogram with the iodine-azide reagent gave the anticipated reactions of a sulfur-containing amino acid in this position. Beta-aminoisobutyric acid has also been reported to have the same Rf values as methionine sulfoxide (Crumpler, Dent, Harris and Westall, 1951), but as this compound has never been identified as a protein hydrolysate product, it is unlikely that this amino acid is X_1.

A second unknown substance, labeled X_2, was characterized by a yellow ninhydrin reaction. Although this reaction is typical of the imino acids, X_2 does not migrate to the site occupied by any of the known substances of this nature.

TABLE II

Nitrogen composition of salivary products of Phormia regina

Product	Dry weight of material analyzed	Total nitrogen	Per cent nitrogen	Mean nitrogen per cent
Secretion collected from salivary glands	1.4 mg.	0.111 mg.	7.9	8.0
	3.6	0.289	8.0	
Secretion collected from puparia (in sand)	2.9	0.231	8.0	7.9
	3.4	0.261	7.7	
	4.8	0.382	8.0	
	4.9	0.388	7.9	
Secretion collected from puparia and dialyzed	7.3	0.732	10.0	10.0
	5.9	0.597	10.0	

A compound with a similar ninhydrin color and Rf values has been reported on chromatograms of free amino acids in potatoes (Dent, Stepka and Steward, 1947), and has also been found free in other dipterous tissues (unpublished data). In the salivary material, this unknown substance is undoubtedly conjugated in the protein molecule, as it appears only after hydrolysis.

In Table II, micro-Kjeldahl nitrogen determinations are compiled. These materials were collected in small aluminum-foil boats, and dried to constant weight before analysis. The nitrogen content of the fluid collected from the glands is in good agreement with that of the material emitted into the sand. The nitrogen values are higher in dialyzed samples, but are still unusually low for a typical protein. Kodani (1948), working with the glandular secretion of *D. melanogaster,* reported 10.8% nitrogen in samples exhaustively extracted with ether, and further demonstrated that a considerable quantity of crystalline, inorganic salt was present after hydrolysis and evaporation of HCl from the residue. He proposed that the low nitrogen figures could be accounted for by the presence of the salt together with a large amount of glutamic acid and glucosamine, both of which are low in

nitrogen. The *Phormia* products, when permitted to dry on glass slides, frequently crystallized in dendritic patterns which could be a result of the presence of inorganic salts. However, if this were the principle contributing factor to the low nitrogen figures, it would be expected that dialyzed samples would contain more nitrogen than the determined values of 10%. Lesher (1952) presented data suggesting that the substance synthesized by the larval salivary gland of *Drosophila robusta* is a conjugated protein composed of a protein bonded to a polysaccharide, *i.e.,* a mucopolysaccharide. This could possibly explain the low nitrogen figures obtained by Kodani and in this study.

Biuret and ninhydrin (triketohydrindene hydrate) tests were both strongly positive and indicative of the proteinaceous nature of the salivary gland secretion. The protein was water soluble and could be precipitated with hot or cold 10% trichloroacetic acid. Millon's reaction was positive, confirming the presence of tyrosine, and the xanthoproteic test also gave a strong reaction. The Hopkins-Cole test for tryptophane was slightly positive, but as only acid hydrolysates were prepared, the presence of this amino acid was undetected. Although glucosamine was demonstrated as a constituent of the protein, results of the Molisch test were doubtful. Specific carbohydrate tests, Benedict's, Barfoed's and Selivanoff's, were all negative.

Summary

1. In a comparative chromatographic study of the fluid in late larval salivary glands, and the substance which is responsible for adhering puparia of *Phormia regina* to their substrate, evidence is presented indicating that these materials are identical in nature and composition; this constitutes convincing proof that the "puparial cement" is the ultimate fate of the larval salivary secretion.

2. The identity of these secretions, collected from two different sites (the salivary glands and the exterior of the puparia), has been demonstrated by like nitrogen composition; the presence of a single free amino acid, lysine, in each fluid; the same components in the protein constituent (amino acids—glycine, alanine, serine, threonine, valine, leucine, tyrosine, proline, aspartic acid, glutamic acid, arginine, lysine and cystine; two unknown substances, and the carbohydrate, glucosamine); as well as by the reactions to several biochemical characterization tests.

LITERATURE CITED

Blumel, J., and H. Kirby, 1948. Amino acid constituents of tissues and isolated chromosomes of *Drosophila*. *Proc. Nat. Acad. Sci.,* **34**: 561–566.

Chargaff, E., C. Levine and C. Green, 1948. Techniques for the demonstration by chromatography of nitrogenous lipide constituents, sulfur-containing amino acids, and reducing sugars. *J. Biol. Chem.,* **175**: 67–71.

Consden, R., A. H. Gordon and A. J. P. Martin, 1944. Qualitative analysis of proteins; a partition chromatographic method using paper. *Biochem. J.,* **38**: 224–232.

Crumpler, H. R., C. E. Dent, H. Harris and R. G. Westall, 1951. β-aminoisobutyric acid (α-methyl-β-alanine): A new amino acid obtained from human urine. *Nature,* **167**: 307–308.

Dent, C. E., 1947. The amino-aciduria in Falconi syndrome. A study making extensive use of techniques based on paper partition chromatography. *Biochem. J.,* **41**: 240–253.

DENT, C. E., 1948. A study of the behavior of some sixty amino acids and other ninhydrin-reacting substances on phenol-collidine filter-paper chromatograms, with notes as to the occurrence of some of them in biological fluids. *Biochem. J.*, **43**: 169–180.

DENT, C. E., W. STEPKA AND F. C. STEWARD, 1947. Detection of the free amino acids of plant cells by partition chromatography. *Nature*, **160**: 682–683.

EPHRUSSI, B., AND G. W. BEADLE, 1936. A technique of transplantation for *Drosophila*. *Amer. Nat.*, **70**: 218–225.

FRAENKEL, G., AND V. J. BROOKES, 1953. The process by which the puparia of many species of flies become fixed to a substrate. *Biol. Bull.*, **105**: 442–449.

KODANI, M., 1948. The protein of the salivary gland secretion in *Drosophila*. *Proc. Nat. Acad. Sci.*, **34**: 131–135.

LACOUR, L. F., AND R. DREW, 1947. Partition-chromatography and living cells. *Nature*, **159**: 307–308.

LESHER, S. W., 1952. Studies on the larval salivary gland of *Drosophila*. III. The histochemical localization and possible significance of ribonucleic acid, alkaline phosphatase and polysaccharide. *Anat. Rec.*, **114**: 633–652.

WILLIAMS, R. J., AND H. KIRBY, 1948. Paper chromatography using capillary ascent. *Science*, **107**: 481–483.

YASUZUMI, G., AND G. MIYAO, 1950. A qualitative analysis of the amino acids in isolated chromosomes. *Exp. Cell. Res.*, **2**: 153–157.